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Healing activity induced by Cramoll 1,4 lectin in healthy and immunocompromised mice

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ABSTRACT

Cramoll 1,4 is a lectin extracted from seeds of *Cratylia mollis* Mart. Many assays have shown the cytokine release activity and pro-inflammatory profile of this lectin. Here, we used Cramoll 1,4 in the treatment of cutaneous wounds in normal and immunocompromised mice for available your cicatricial power. Surgical wounds were treated daily with a topical administration of Cramoll 1,4 and parameters as edema, hyperemia, scab, granulation and scar tissues as well as contraction of wounds were analyzed. Cramoll 1,4 wounds showed higher edema and arrival of more polimorphonuclear cells at the site of lesions. Granulation tissue and collagen fiber deposition were observed with higher intensity in all Cramoll 1,4 treated wounds and promoted excellent closing and repair of lesions in less time than other groups. Results showed that Cramoll 1,4 lectin was effective in the repair of experimental lesions in mice and can be used as a future cicatricial compound.

1. Introduction

The healing process aims to recover anatomical and physiological integrity of tissue. The repair of a wound is a complex sequence of biochemical and cellular events in response to tissue lesion (Stadelmann et al., 1998). After injury, blood vessels are disrupted resulting in extravasation of blood components such as proteins, coagulation factors and platelets. These latter components promote platelet aggregation and blood coagulation for the re-establishment of homeostasis. To the inflammatory site, neutrophils and macrophages are attracted. These immune cells act by phagocytosing remaining cells, fibrin and damaged extracellular matrix and opportunistic pathogen bacteria infecting the wound site (Kapoor and Appleton, 2005). As part of the sequence, the migration of inflammatory cells and regeneration of remaining blood vessels occur characterizing a new tissue in formation, the granulation tissue. Finally, fibroblasts arrive at the wound site to fill the place with collagen matrix and to promote contraction of the injury (Werner and Grose, 2003).

Many studies use immunosuppression to elucidate the biochemical stages involved in the repair process and, mainly, the immune response of the organism to injury. The drugs cyclosporine and methotrexate (Ciesielski et al., 1998; Koning et al., 2006) are immunosuppressant drugs commonly used in these studies by function of its action mechanism that involves the blockage and inactivation of some cytokines, growth factors, integrins and polimorphonuclear cells (PMN), which allows the shortening of some stages of the cicatricial process (Peters et al., 2006).

A great number of lectins with distinct characteristics and specificity have been used for their immunomodulatory activity, lymphoproliferation, CD4-mediated signal transduction and functional activation of monocytes and macrophage-like cells (Lee et al., 2007; Tamma et al., 2003).

Cramoll 1,4 is a lectin extracted from seeds of *Cratylia mollis* Mart., a plant native to Northeastern Brazil. Cramoll is from the

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same Leguminosae family and the same Diocleinae subtribe as Concanavalin A lectin (*Canavalia ensiformis*) and like Con A, Cramoll is specific for glucose/mannose. Four multiple forms have been purified from this plant – Cramoll 1, Cramoll 2, Cramoll 3, Cramoll 4 and preparations containing multiples 1 and 4 form associates, called Cramoll 1,4 (Correia and Coelho, 1995). Several studies have demonstrated the immunomodulatory profile of this lectin, production of IFN-γ and nitric oxide (Melo et al., 2010a), mitogenic activity on human lymphocytes (Maciel et al., 2004) and antitumor activity (Andrade et al., 2004).

This study investigated, *in vivo*, clinical and histopathological aspects of cutaneous wounds performed experimentally in healthy and immunocompromised mice, aiming to find an alternative biomaterial for injury repair.

2. Materials and methods

2.1. Animals

Females of albino Swiss mice ($n = 30$/group), 12 weeks old and 45.0 ± 2.0 g were raised at the animal facilities of Laboratório de Imunopatologia Keizo Asami – UFPE. Each animal was maintained in an individual cage, under controlled environmental conditions (12-h light/dark cycle, temperature $23 \pm 2\,^{\circ}C$ and humidity $55 \pm 10\%$) with water and commercial food *ad libitum* (Labina®, Agribrands of Brazil). All mice were treated and sacrificed in accordance with the Ethical Committee of Universidade Federal Rural de Pernambuco for Experiments with Laboratory Animals (Ministry of Health, Brazil, 012/02).

2.2. Lectin extraction and purification

C. mollis seed extract (10% w/v prepared in 0.15 M NaCl) was fractionated using ammonium sulphate (40–60% w/v) and the fraction obtained was submitted to affinity chromatography in Sephadex G-75. Cramoll 1,4 preparation was bioselectively eluted with 0.3 M D-glucose in 0.15 M NaCl, dialyzed against 0.15 M NaCl during 24 h and lyophilized (Correia and Coelho, 1995).

2.3. Immunosuppression induction

Methotrexate (MTX) was administered to each animal using a low-dose (0.8 mg/kg/week). MTX was administered, according to Ciesielski et al. (1998), intramuscularly in 0.15 M NaCl weekly at 7 days before surgery, on surgery day and 7 days after surgery.

2.4. Experimental protocol and groups

Animals were divided into four groups ($n = 30$/group) and were anesthetized for the surgical procedure using 2% xilazine chloridrate (10 mg/kg) and 10% ketamine chloridrate (115 mg/kg) in subcutaneous injections (Hall and Clarke, 1991). Each animal was placed in a prone position and prepared for aseptic surgery using 2% chlorhexidine digluconate. A standard wound (0.5 cm of diameter) was performed on the dorsal thoracic region using scalpel and curved blade surgical scissors by removal of epidermal and dermal layers with minimal bleeding. Each wound was treated daily with 100 μL of solution, as follows: (1) Cramoll – healthy animals topically treated with Cramoll 1,4 lectin, in 100 μg/mL dose; (2) control – healthy animals topically treated with 0.15 M NaCl; (3) Cramoll Im – immunocompromised animals topically treated with Cramoll 1,4 lectin, in 100 μg/mL dose and (4) control Im – immunocompromised animals topically treated with 0.15 M NaCl. The clinical characteristics of the experimental wounds were observed every day, considering the following aspects: edema, hyperemia, secretion, scab, granulation, epithelialization and scar tissues (Fig. 1). On a daily basis, wound areas were measured using a pachometer and were calculated as follows: $A = \pi \times R \times r$, where A, R and r are mean area, large ray and small ray, respectively (Prata et al., 1988). At each time of biopsy, on the 2nd, 7th and 12th days after surgery, 10 animals were drawn from the experimental groups, subjected to subcutaneous anesthesia and euthanized by cervical disruption. After this procedure, the skin around the area of the wound was removed, extending one centimeter beyond each dorsal and ventral edge, and internally until the muscular layer. Immediately after the withdrawal of skin, the samples were transferred to filter paper and incubated in formaldehyde (4%, v/v) in 0.01 PBS M, pH 7.2 for a maximum period of 48 h for the processing of histological sections.

2.5. Microbiological evaluation

Microbiological evaluation was carried out using "swabs" in the injury area at the moment of surgery and the respective days of biopsies. This routine evaluation was done to evaluate the degree of contamination of injuries. The microorganisms were classified by the morphological aspects of colonies and Gram-staining pattern.

2.6. Statistical analysis

Data were analyzed using non-parametric tests. To detect differences between groups, the Mann–Whitney U test was used. All results were expressed as mean values of groups ± standard deviation and were analyzed considering the value of $p < 0.05$ as statistically significant.

3. Results

3.1. Cramoll 1,4 promoted more PMN in inflammatory site during inflammatory phase

Inflammatory parameters such as edema and hyperemia were observed in all experimental groups. Edema was observed for 6 days and was most intense in Cramoll 1,4 treated wounds for both edema on edge and at center of lesions (Fig. 2A and B, respectively). In fact, Cramoll wounds showed higher edema on edge formation in relation to the control group, but Cramoll Im showed lower values in relation to its respective control (control Im group) (Fig. 2A). Cramoll also presented higher values for edema at center of lesions in relation to the control (Fig. 2B). Wound hyperemia was observed in all groups, showing pale aspect (Fig. 3a–d). In relation to inflammatory infiltrate and PMN, a deficiency or reduction of cells in the site of injuries was observed in immunocompromised animals, in relation to health animals. Wounds of Cramoll group revealed more PMN in the inflammatory site of the wounds than its respective control (Fig. 3e and f) and this result was followed by Cramoll Im and control Im groups (Fig. 3g and h).

3.2. Most collagen fibers were observed in wounds treated with Cramoll 1,4 at fibroplasia phase

Granulation tissue, which consists of blood tissue under a crust, was observed in all experimental wounds. In fact, granulation tissue was observed on lesions from the 4th day until 12th day of assay on and above skin (Fig. 4A and B). Cramoll wounds showed more prevalence of granulation tissue than its respective control (Fig. 4A) and control Im group showed more granulation tissue than the Cramoll Im group (Fig. 4B).

Crust formation occurs when plasma exudates present on lesion surface dry in the external environment. Crust presence was observed on all experimental days. However, only Cramoll group showed higher and statistical ($p < 0.05$) crust presence in relation

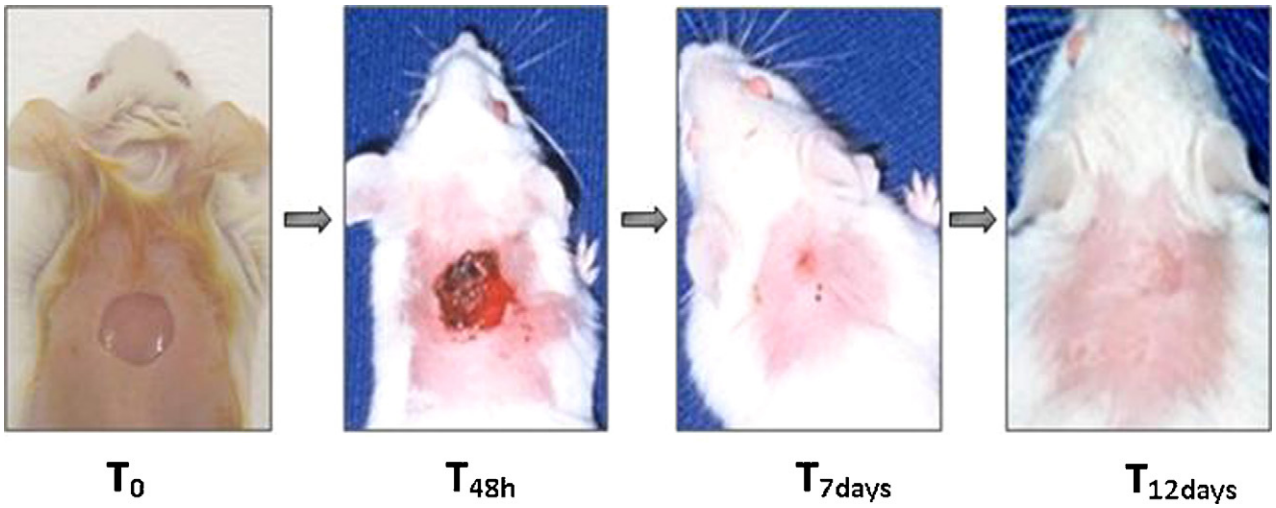

Fig. 1. Scheme of wound production. At T_0 time we performed surgical wounds made aseptically (using 2% chlorhexidine digluconate and iodopovidone) and using a 1 cm^2 guide. At 48 h granulation tissue and scab presences on lesion area can be observed. Seven days after surgery the formation of cicatricial tissue and presence of the scar can be observed in some mice by the 12th day. Clinical and microbiological parameters were available at four experimental times and contraction of lesion area was measured using a pachometer.

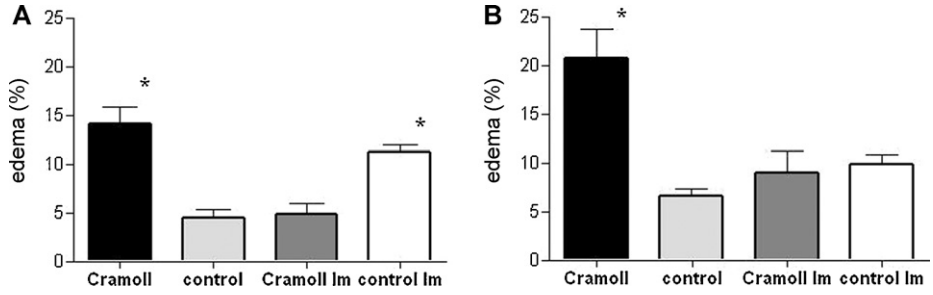

Fig. 2. Presence of edema in wounds of experimental groups observed in experimental wounds until the 6th day. (A) Edema on lesion edge showed higher values in the Cramoll group in relation to the control and in the control Im in relation to the Cramoll Im group. Edema of the Cramoll group was also superior to the Cramoll Im. (B) Edema on center of lesions. The Cramoll group was also superior to its respective control. $^*p < 0.05$.

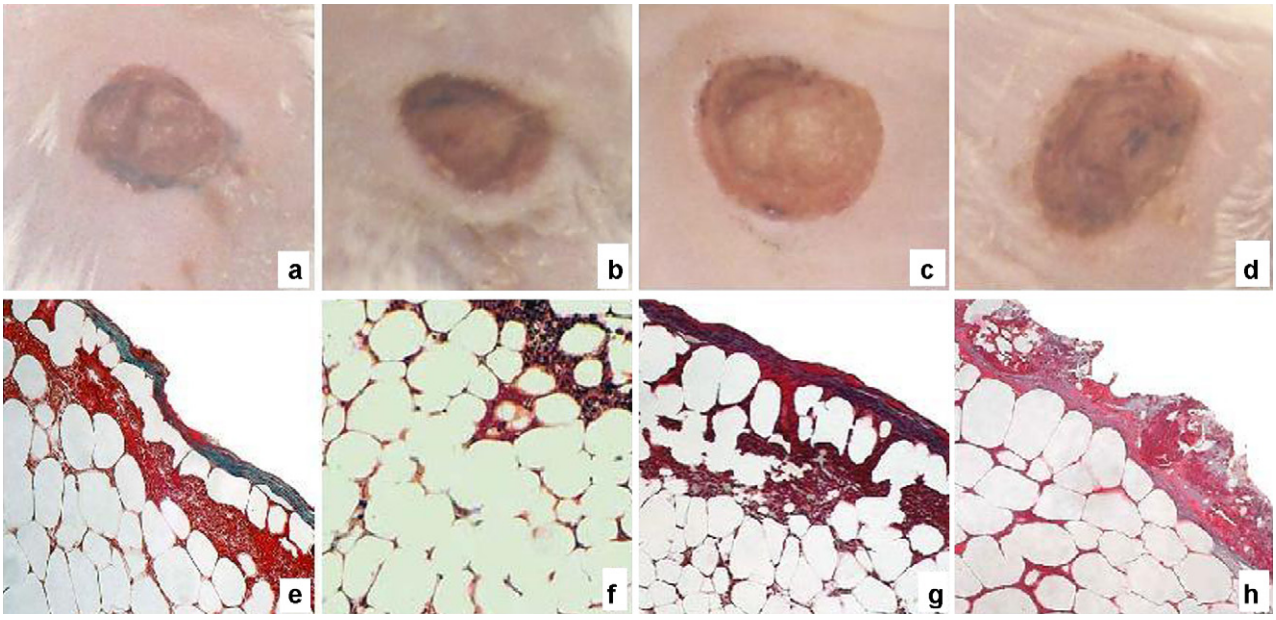

Fig. 3. Scheme summarizing macroscopic lesions (a–d) and histopathological aspects (e–h) of the inflammatory phase (biopsy time – 2nd day after surgery) in experimental wounds. (a–e) shows macroscopic lesions and the pale aspect of hyperemia observed for all experimental groups. (a and e) Cramoll group; (b and f) control group; (c and g) Cramoll Im group and (d and h) control Im group. Cramoll treatment showed higher levels of PMN in the lesion site at 48 h than the control groups. Optical microscopy (200×).

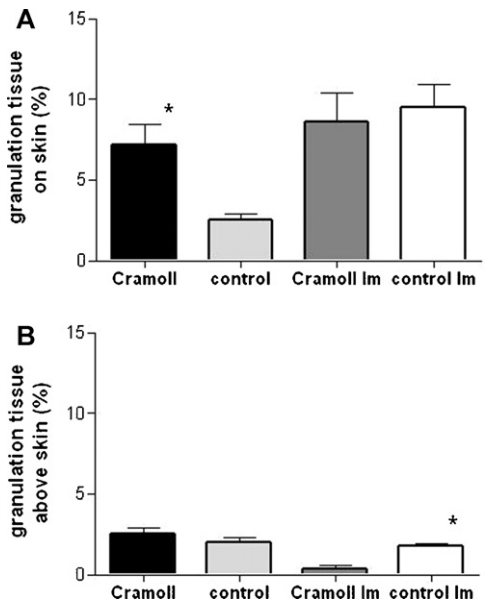

Fig. 4. Presence of granulation tissue on all experimental wounds. Cramoll and control wounds showed granulation tissue from the 4th until 12th day of assay. (A) Granulation tissue on skin. Cramoll showed more granulation tissue than its respective control and other groups were equivalent among them. (B) Granulation tissue above skin. The control Im group was the only group that showed more granulation tissue than the Cramoll Im group. *$p < 0.05$.

to the control (13.1 ± 7.02 and 5.4 ± 3.3 for Cramoll and control groups, respectively).

In the fibroplasia phase it could be observed, through histopathological samples taken the 7th day by biopsy, that Cramoll injuries demonstrated elevated collagen deposition (Fig. 5). This aspect experienced progressive reduction in the Cramoll Im group and only the control group showed meaningful collagen deposition (Fig. 5e–h).

3.3. Maturation phase showed higher healing activity promoted by Cramoll 1,4 treatment

In experimental mice, closing of all injuries was observed through pachometer measurement on each wound over 12 days. Results showed that cicatricial tissue was observed in wounds of the 5th day until the 12th day. However, 90% of wounds were closed by the 10th day for the Cramoll group (Fig. 6). Control Im and Cramoll Im closed 90% of wounds on the 11th day and the control closed on the 12th day of assay. The histopathological slices differed from the macroscopic aspect of injuries shown in Fig. 7a–d. The injuries of the Cramoll group, besides closing before its respective control group, demonstrated an excellent repair in relation to the deposition of collagen and beginning of development of annex sprouts (Fig. 7e). Cramoll was the only group that presented this characteristic. The control group also showed collagen deposition and re-epithelization (Fig. 7f). The Cramoll Im group also showed higher collagen deposition, but did not show annex sprouts (Fig. 7g). The control Im group had re-epithelization, but the histopathological slices demonstrated that it was deposited as a matrix poor in collagen fibers in the wound site that conferred local tension fragility and inefficient tissue repair (Fig. 7h). The decrease of collagen deposition in the maturation phase can be explained by the deficient arrival of fibroblasts in the injury area over 7 days. In fact, in the control Im group it was observed that the fibroplasia phase started before the end of the delayed inflammatory phase.

3.4. Cramoll group did not show contamination of experimental wounds

The microbiological analysis of injuries showed that all the groups were contaminated over 12 days, except animals in the Cramoll group that had no incidence of microorganisms in their injuries. The control group showed higher contamination of *Staphylococcus* sp. and *Micrococcus* sp. at all experimental biopsy times (20.5 ± 7.3; 14 ± 4.1; 11.2 ± 2.5, for 48 h, 7 days and 12 days, respectively) and this result was followed by Cramoll Im at surgery time ($T_0 - 10 \pm 3.4$) and on the 12th day (13.3 ± 2.8) and control Im on the

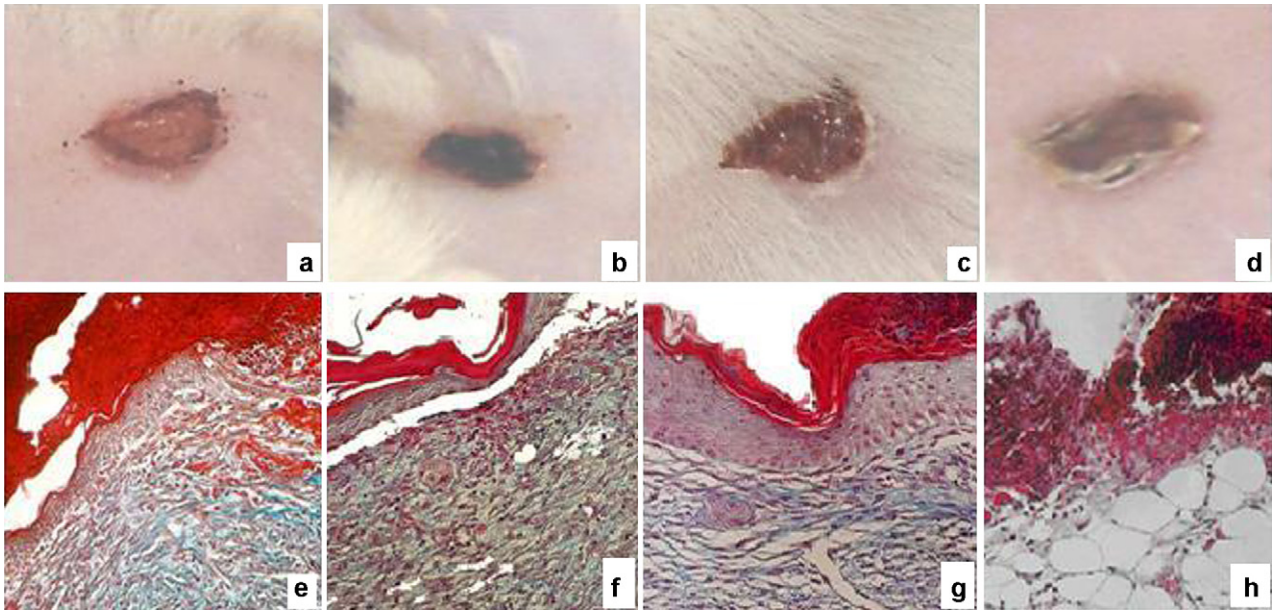

Fig. 5. Scheme summarizing macroscopic lesions (a–d) and histopathological aspects (e–h) of the fibroplasia phase (biopsy time – 7th day after surgery) from experimental wounds. Crust presence was also observed. (a and e) Cramoll group; (b and f) control group; (c and g) Cramoll Im group and (d and h) control Im group. Cramoll wounds demonstrated elevated collagen deposition (fibers in blue color) and control Im was the only group that showed a delay of collagen deposition. Optical microscopy (200×).

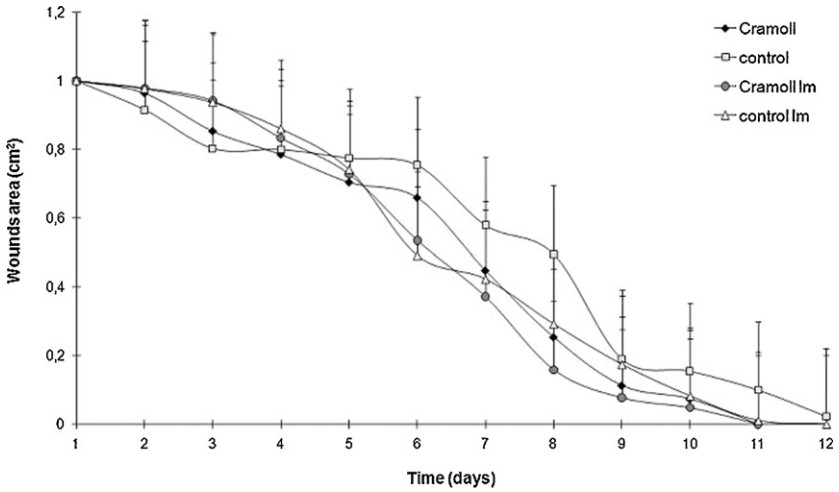

Fig. 6. Measurement of wound area reduction, by pachometer, of experimental wounds during 12 days. It was observed that 90% of wounds were closed by the 10th day for the Cramoll group. Control Im and Cramoll Im closed 90% of their wounds by the 11th day and the control closed on the 12th day of assay. Results are expressed as mean ± SD and p value for Cramoll group was less than 5% in relation to control.

7th day (7.5 ± 3.1; 3.7 ± 2.1; 3.3 ± 1.4, for 48 h, 7 days and 12 days, respectively). Statistical values ($p \leq 0.05$) were observed surgery time for control in relation to Cramoll and Cramoll Im in relation to others groups; control in relation to others groups at 48 h of biopsy; control and control Im in relation to respective treatments at 7 days of biopsy and control and Cramoll Im in relation to Cramoll and control Im groups, respectively.

4. Discussion

Lectins have been used as biomaterials to modulate the biological response, activating cells of the immune system (Alencar et al., 2003; Cavada et al., 2001), enlisting neutrophils through indirect mechanisms (Assreuy et al., 2003), promoting pro-inflammatory effects in PMN and inducing the release of cytokines (Alencar et al., 2005; Cavada et al., 2001) as well as triggering the proliferation of fibroblasts (Sell and Costa, 2003). Mistletoe, Jacalin, Abrin, Con A and PHA lectins induce T cell activation, cytokines release (Heiny and Beuth, 1994) and still modulate the Th1/Th2 immunological response balance (Bhutia et al., 2009; Lyu and Park, 2006; Pani et al., 2000; Wimer, 1990).

Previous assays accomplished by our group have shown a potential pro-inflammatory and immunomodulatory activity induced by Cramoll 1,4 lectin. Lymphocyte cultures *in vitro* stimulated by this lectin showed increased reactive oxygen species and cytosolic calcium and also expressed IL-1β cytokine by RT-PCR analysis (Melo et al., 2010b). Other assays have demonstrated the higher production of IFN-γ and nitric oxide production on *in vitro* cultures stimulated with Cramoll 1,4 lectin (Melo et al., 2010a). Recent assays also demonstrated higher proliferative induction promoted by this lectin, in addition to IL-2, IL-6, nitric oxide and NK cell activation, in pre-immunized mice with Cramoll 1,4 (Melo et al., 2010c).

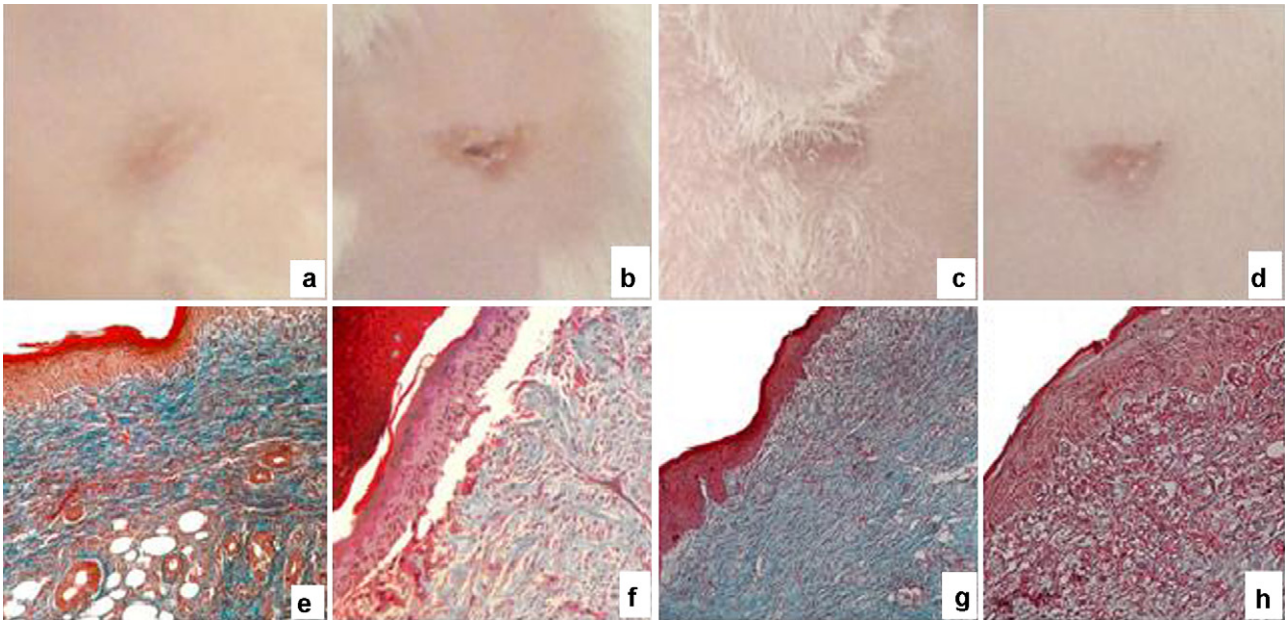

Fig. 7. The maturation phase and total closing of experimental lesions with biopsy time on the 12th day after surgery. Macroscopic aspects of lesions (a, b, c and d) and histopathological aspects (e, f, g and h). (a and e) Cramoll group; (b and f) control group; (c and g) Cramoll Im group and (d and h) control Im group. Cramoll wounds showed total closing before other groups and this repair demonstrated development of annex sprouts. Cramoll Im also showed higher collagen deposition and control Im group showed a matrix poor in collagen fibers in the wound site that conferred local tension fragility and inefficient tissue repair. Optical microscopy (200×).

Here, Cramoll 1,4 was used on wounds of health and immunocompromised mice.

Inflammatory signals of wound healing, such as edema, hyperemia and the arrival of PMN at the lesion site, could be observed in our results. The Cramoll group showed the most edema and PMN prevalence of all groups and this result was shown by higher protein concentration at the site of the experimental lesions. This higher protein accumulation, in addition to the higher metabolism induced by PMN to remove cell debris and opportunistic microorganisms, induces higher osmotic pressure in the wound and attracts plasma, inducing edema formation. On the other hand, PMN reduction in the site of injuries of the immunocompromised mice can be explained as the result of the immunossupressive drug used, methotrexate, which directly affects the subunit CD18 molecule that constitutes β2 integrin of the lymphocyte cellular surface (Ciesielski et al., 1998). The β2 integrin is necessary for normal development of the inflammatory response. Deficiency of the CD18 subunit did not induce leukocyte migration to tissues due to lack of a firm adhesion to the endothelium and, as a result, it causes a reduction in site inflammation (Sindrilaru et al., 2006).

Collagen deposition in the fibroplasia phase is required for the efficient arrival of fibroblasts to the wound site. This aspect could be observed in wounds of the Cramoll group that also showed efficient repair. However, collagen deposition observed on immunocompromised mice was deficient and suffered progressive decrease. This result also can be explained by treatment with methotrexate. Peters et al. (2006) observed that CD18 present in the neutrophil surface during migration emits a chemical signal that induces infiltrations of macrophages to secrete TGF-β1. Therefore the lack of CD18 in one or another cell leads to an extremely reduced release of TGF-β1 due to defective adhesion and to subsequent extravasation of the phagocyte in the injury area. Ronty et al. (2006) additionally affirmed that this deficient release of TGF-β1 promotes a delay in the arrival in fibroblasts to the injury site with consequential deficit collagen staple fiber deposition.

One of the final and important stages in the cicatricial process is characterized by total closing of the injury that not only involves deposition of collagen matrix (fibers of type III), but also the total re-epithelization of the wound from centripetal differentiations of basal epithelial cells, the keratinocytes, that are disposed at the edge of lesion, next to center of injury, increasing wound tensile strength (Pilcher et al., 1999). The Cramoll group was the only group that induced efficient closure of lesions with the presence of annex sprouts. The Cramoll Im group also closed its lesions with efficient collagen fibers deposition. However, the control Im group showed a matrix poor in collagen fibers in the wound site that conferred local tension fragility and inefficient tissue repair. Similar results were described by Zweers et al. (2007), who observed that keratinocytes do not depend on adhesion or migration of collagen for the closing of injury, yet the absence of this protein promoted a reduction of the tension of wound, with a change in the organization of extracellular matrix. These studies can explain the closing of the control Im wounds without efficient repair.

If the wounds are not well treated, they can be infected. Infected wounds heal more slowly, re-epithelialisation is more prolonged, and there is also the risk of systemic infection (Inngjerdinger et al., 2004). In our study we could observe bacterial contamination of wounds, but this contamination was induced by normal flora, such as *Staphylococcus* sp. and *Micrococcus* sp. Because of this, we could not observe secretion and excessive exudates in the lesion area. Interestingly, Cramoll wounds did not show any contamination at all experimental times.

Other natural compounds have been used as wound healing agents. Chitosan, the principal component of exoskeletons of crustaceans and insects, accelerate wound healing, decrease treatment frequency, and give comfortable and painless wound surface protection (Kojima et al., 2001; Senel and McClure, 2004). A Tibetan mushroom formed by a symbiotic association of yeasts and lactic acid bacteria was capable of inhibiting paw edema in rats (Diniz et al., 2003). The importance of glycoproteins (including lectins), as components of *Aloe vera* extract gel, has been asserted for promoting wound, burn and frost-bite healing, and for showing anti-inflammatory and antifungal properties (Choi and Chung, 2003). Sell and Costa (2002) also described an improved effect of PHA lectin in the skin tissue repair process of Wistar rats when compared to *Triticum vulgaris* (WGA) and *Artocarpus integrifolia* (jacalin) lectins.

Although the exact mechanism of the immunomodulatory action of Cramoll 1,4 is not totally known, Cramoll 1,4 possibly induces the activation of T lymphocytes through transmembrane signals, similar to Con A (Hadden, 1988). In fact, studies have affirmed that lectin binding to glycans of the cell surface can cluster target molecules, a pivotal step for initiating cellular signaling pathways (Brewer, 1996; Gabius, 2001; Villalobos and Gabius, 1998).

Our results showed that Cramoll 1,4 lectin was effective for the repair of experimental lesions in mice and can be used as a cicatricial compound in the future.

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